Neural Modulation of Cephalexin Intestinal Absorption Through the Di- and Tripeptide Brush Border Transporter of Rat Jejunum In Vivo

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ABSTRACT

Intestinal absorption of β-lactamine antibiotics (e.g., cefixime and cephalexin) has been shown to proceed through the dipeptide carrier system. In a previous study, nifedipine (NFP), an L-type calcium channel blocker, enhanced the absorption of cefixime in vivo but not in vitro, and it was suggested that neural mechanisms might be involved in the effect of NFP. The aim of the present study was to assess the involvement of the nervous system on the intestinal absorption of cephalexin (CFX). To investigate this, we used a single-pass jejunal perfusion technique in rats. NFP and diltiazem enhanced approximately 2-fold the plasma levels of CFX in treated rats versus untreated controls. NFP also increased approximately 2-fold the CFX level in portal plasma and increased urinary excretion of CFX, thus indicating that CFX did effectively increase CFX intestinal absorption. Perfusing high concentrations of dipeptides in the jejunal lumen competitively reduced CFX absorption and inhibited the enhancement of CFX absorption produced by NFP. Hexamethonium and lidocaine inhibited the effect of NFP, whereas atropine, capsaicin, clonidine, and isoproterenol enhanced CFX absorption by the same order of magnitude as NFP. Thus, complex neural networks can modulate the function of the intestinal di- and tripeptide transporter. Sympathetic noradrenergic fibers, intestinal sensory neurons, and nicotinic synapses are involved in the increase of CFX absorption produced by NFP.

Peptidomimetic drugs, such as the orally active β-lactamine antibiotics, cross the intestinal mucosa through the di- and tripeptide transporter and by additional mechanisms such as passive diffusion. Previous reports from our laboratories have shown that the bioavailability of amoxicillin (Westphal et al., 1990) and cefixime (Duverne et al., 1992) is increased by nifedipine in humans. Investigation in the rat of the mechanism involved showed that this effect of nifedipine (NFP) could be demonstrated only in vivo, and probably involved neural pathways (Harcouët et al., 1997).

The present experiments were undertaken to further analyze this mechanism. Because we were especially interested in the possible control of the proton-dependent di- and tripeptide transporter, we chose to use as a test molecule, cephalexin (CFX). This cephalosporin is reputed to be mainly carried by the dipeptide transporter and to present, in addition, only a small passive entry in the mammalian intestine (Bai et al., 1992).

CFX absorption was measured in vivo in the rat by measuring CFX blood levels during jejunal perfusion of the drug; the effect of NFP on CFX absorption was measured, and its mechanism was investigated by using several agonists and antagonists of neural receptors.

Materials and Methods

Surgical Preparation

All studies were carried out in male Wistar rats (240–260 g) (Iffa Credo, Les Oncins, F-69210 L’Arbresle, France) fasted 18 h with water ad libitum. The rats were anesthetized with ethylurethane (1.215 g/kg, i.m.; Prolabo, Paris, France). Normal body temperature was maintained with a heating device.

A laparotomy was performed, and the bile duct was ligated to prevent possible enterohepatic recycling of CFX. Approximately 1 cm proximal to the ligament of Treitz, the duodenal contents were diverted through a glass cannula (4 mm o.d., 2.5 mm i.d., and 20 mm long, bent at 90°) connected to a piece of vinyl tubing (3.69 mm o.d., 2.79 mm i.d., and 10 cm long; Technicon, Tarrytown, NY). Approximately 1 cm aboral from this diversion, an inflow cannula made of Silastic brand tubing (602,175, 1.65 mm o.d., 0.76 mm i.d., and 10 cm long; Dow Corning, Midland, MI) was inserted in the gut. Approximately 10 cm further aborally, a glass outflow cannula identical with

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ABBREVIATIONS: NFP, nifedipine; CFX, cephalexin, KRB, Krebs-Ringer buffer solution; HXM, hexamethonium.

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the duodenal cannula was set up. The jejunal segment was then flushed with saline prewarmed at 37°C to remove residual intestinal contents.

**Arterial Catheter for Blood Sampling.** A short polyethylene catheter (0.58 mm i.d. × 0.965 mm o.d., and length 15 mm; Clay Adams PE 50, Parsippany, NY) continued by a Silastic catheter (0.64 mm i.d. × 1.19 mm o.d., and length 15 cm) was inserted into a carotid artery and secured by a double ligature around the polyethylene part of the catheter. The rats immediately received 250 IU/kg heparin, injected through the carotid catheter as soon as it was secured. The catheter was then filled with saline and clamped. Blood samples (0.6 or 1.5 ml under stimulated or basal conditions, respectively) were collected in Eppendorf 1.5 ml centrifuge tubes (Eppendorf, Fremont, CA) containing 2 mg EDTA/ml whole blood, after having discarded the first drop corresponding to the catheter dead space. Blood samples were collected before the CFX perfusion and 15, 30, and 60 min thereafter. To compensate for blood loss, an equal volume of Hæmaccel (Behring, Marburg, Germany) was injected through the carotid catheter after each blood sampling. After 3 min centrifugation at 7000g (Biofuge 13; Heraeus, Les Ulis, France), the plasma samples were collected and stored at −20°C until the CFX assay.

**Portal Catheter for Blood Sampling.** A polyethylene catheter (0.30 i.d. × 0.70 mm o.d., and length 45 mm; Clay Adams PE 10) was inserted into the portal vein. It was previously connected to another 15-cm polyethylene catheter (0.58 i.d. × 0.965 mm o.d.; Clay Adams PE 50). The rats immediately received 250 IU/kg heparin, injected through the portal catheter as soon as it was secured. The catheter was then filled with saline. The portal catheter was connected to a syringe to allow blood sampling. Portal blood was collected, centrifuged, and the plasma was stored as described above.

**Truncal Vagotomy.** In six rats, cervical bilateral truncal vagotomy was carried out 30 to 60 min before beginning the intraluminal perfusion.

**Collection of Urine.** Before the perfusion experiment and after the jejunal cannulation, the bladder was emptied and the urethra was ligated. At the end of the intestinal perfusion, the bladder was clamped and its contents were collected.

**Perfusion Technique**

Perfusion solutions were injected with a peristaltic pump (Minipuls II; Gilson Co, Paris, France) through an inlet tubing water-jacketed at 37°C before its entry in the jejunal segment. The flow rate was 4 ml/15 min. The jejunal segment was routinely prewashed with 9 g/liter NaCl prewarmed at 37°C before beginning the intraluminal perfusion.

**Solutions Used for Jejunal Perfusion**

The Krebs-Ringer buffer solution (KRB), pH = 7.5, contained MgCl₂/₆H₂O (0.5 mM), KCl (4.5 mM), NaCl (120 mM), Na₂HPO₄ (0.7 mM), NaH₂PO₄ (1.5 mM), CaCl₂ (1.2 mM), NaHCO₃ (15 mM), and 20 mM of the following treatments as an i.v. bolus: propranolol (1 mg/kg; Sigma), prazosin (0.5 mg/kg; Sigma), idazoxan (0.3 mg/kg; Reckitt and Colman, Kingston Upon Hull, England), clonidine (100 or 300 μg/kg; Catapressan 0.15 mg/ml, Boehringer Ingelheim, Paris, France), NFP (2 mg/kg; 0.5 mg/ml solution in 10% ethanol), atropine (250 μg/kg; Sigma), or yohimbine (1 mg/kg; Sigma).

The following agents were administered as a 75-min i.v. infusion during the jejunal perfusion of NFP or placebo and during the perfusion of CFX: isoproterenol (0.1–0.3 or 3 μmol/kg/h; Sigma), phenylephrine (100 μg/kg; Neosynephrine Badrial, Boehringer Ingelheim), hexamethonium (HX; 6.7 mg/kg/h following a primo bolus of 6.7 mg/kg, Sigma).

Finally, the following substances were administered mixed with 9 g/liter NaCl, NFP, and CFX in the jejunal perfusion fluids: lidocaine (0.1mg/ml; Xylocaı ¨ne 1%, Astra, Nanterre, France,) during the whole 90-min perfusion period, capsaicin (100 μg/kg at a concentration of 44 μM, Sigma), or a dipeptide mixture of 85 or 170 μM concentration containing Gly-Gly 80 or 160 mM and Gly-Pro 5 or 10 mM (Sigma) during the 75 min of NFP or placebo and of CFX perfusion.

**Analytical Method**

The CFX concentration in plasma and urine was determined by HPLC: 200 μl of plasma, 40 μl of water, and 300 μl of acetonitrile (Merck) were mechanically mixed. The mixture was centrifuged at 6700g for 5 min; 4 ml of dichloromethane (Merck) was added to the supernatant and the mixture was shaken during 15 min; 50 μl of the aqueous supernatant was injected into the chromatograph. Urine was only diluted with distilled water before injecting the sample into the chromatograph.

Chromatographic separation was performed on a Supelcosil LC18 column, 250 × 4.6 mm, 5 μm (Supelco, Sigma Aldrich, St. Quentin Fallavier, France). The apparatus was composed of a SPD-6AV pump (Shimadzu), a WISP 712 automatic sampler (Waters, Paris, France), a SPD-6AV UV detector (Shimadzu) and an integrator (Shimadzu). The flow rate was 1.5 ml/min. The mobile phase was a mixture of sodium acetatebuffer 0.01 M, pH = 5.2 (Normapur, Prolabo) and acetonitrile (94.5/4.5, v/v).

**Statistical Analyses**

The results are expressed in the figures and table as means ± SEM. They were compared by ANOVA, followed when necessary by a multigroup comparison test (Dunnett’s or Scheffe’s) or by the nonparametric Mann-Whitney’s U test when the variances of the groups compared were different. p < .05 was considered significant.

**Results**

Figure 1 illustrates the time course of CFX concentration in arterial plasma. The CFX concentration after 60 min of intestinal perfusion was approximately twice as large when the CFX perfusion was preceded by NFP treatment (4.30 ± 0.60 mg/liter versus 2.16 ± 0.68 mg/liter; p < .05).

As expected, the measurement of portal CFX showed that CFX concentration was approximately twice as large in the portal blood than in the peripheral arterial blood. When the CFX perfusion was preceded by NFP, the portal plasma CFX level increased approximately 2-fold with respect to the group receiving CFX alone (p < .05). Because the effect of NFP was identical when measuring CFX in arterial or in portal plasma, we decided to use the simpler method of arterial blood sampling for further investigations.

To determine whether the action of NFP is local on the jejunal mucosa or systemic, we compared the effect of NFP when it was administered either intraluminally in the jejunal lumen or systemically by bolus venous injection. The plasma CFX concentration increased in the same fashion when NFP was perfused in the jejunal or injected in the
saphenous vein (4.30 ± 0.60 mg/liter versus 4.60 ± 1.30 mg/liter after 60 min, respectively, N.S.).

To investigate whether increased absorption or decreased urinary elimination of CFX was involved in the effect of NFP, we measured the elimination of CFX in urine during the experiment. The amount of CFX measured in urine during the 60 min of CFX intrajejunal perfusion was in fact much larger when CFX was preceded by intrajejunal NFP (60.3 ± 20.1 mg/60 min) than the amount of urinary CFX measured after perfusion of CFX alone (12.5 ± 7.3 mg/60 min, p < .01). This finding indicates that the effect of NFP was not due to a decrease of urinary excretion.

To determine whether the effect of NFP was correlated to the calcium channel blocking activity of this drug, or to some unrelated property pertaining to its dihydropyridine molecular structure, we replaced the jejunal perfusion of NFP by a perfusion of diltiazem, a drug reputed to block the same L calcium channels than NFP, but which has a different molecular structure. As shown in Fig. 2, diltiazem also increased the plasma concentration of CFX, by 128% after 60 min of CFX perfusion.

To determine the fraction of CFX transport that was ascribable to the di- and tripeptide transporter, we competitively antagonized the di- and tripeptide transporter function by high concentrations (85 Mm and 170 Mm) of neutral dipeptides (Gly-Gly 94% + Gly-Pro 6%) known to cross the apical membrane of enterocytes through the di- and tripeptide transporter. The presence of 85 mM dipeptides in the luminal solution drastically reduced CFX absorption by 63% when CFX was perfused alone (Fig. 3). When CFX was perfused after NFP in the presence of dipeptides, the plasma concentration of CFX was also reduced to approximately the same level (Fig. 3). Increasing dipeptide concentration to 170 mM did not significantly affect the results, suggesting that the residual amount of CFX absorbed under these conditions was likely to use an absorptive pathway other than the di- and tripeptide transporter (diffusion).

Possible neural mediation in the effect of NFP on CFX absorption was investigated by using several agents that act as antagonists at receptors located on nerves or epithelial cells. The intervention of cholinergic receptors and of the vagal pathway was checked first. HXM (6.7 mg/kg + 6.7 mg/kg/h), a nicotinic receptor antagonist, suppressed the effect of NFP on CFX absorption (Fig. 4, *p < .05), but did not change basal CFX absorption in the absence of NFP (Fig. 4). Atropine (250 μg/kg), a muscarinic receptor antagonist, tended to decrease the effect of NFP (Fig. 4), but this was not significant. However, atropine increased CFX absorption when CFX was perfused alone, and this increase was of the same order of magnitude than the increase produced by NFP (Fig. 4, *p < .05). Bilateral acute vagotomy did not affect neither the increase of CFX plasma level produced by NFP (4.09 ± 0.56 mg/liter versus 4.30 ± 0.60 mg/liter at 60 min), nor the absorption of CFX perfused alone (1.41 ± 0.17 mg/liter versus 1.45 ± 0.20 mg/liter).

To assess the involvement of intramural neural fibers and jejunal sensory neurons in the mechanism of the NFP effect
on CFX absorption, we used lidocaine, a local anesthetic, and capsaicin, an agent affecting extrinsic sensory neurons, at a concentration (44 μM) previously shown to stimulate these neurons when administered acutely (Gicquel et al., 1994). Both agents were added to the CFX solution perfused in the jejunum. Lidocaine, which was used at two different concentrations (0.1 and 1 mg/liter) inhibited in a dose-related manner the effect of NFP on plasma CFX (Fig. 5). The largest concentration of lidocaine used completely suppressed the effect of NFP (p < .01), whereas lidocaine suppression did not significantly affect the basal absorption of CFX alone (Table 1). Intraluminal capsaicin increased the plasma CFX level (Fig. 5), thus producing an effect similar to and of the same order of magnitude as that of NFP (plasma CFX at 60 min with capsaicin 5.64 ± 0.59 mg/liter versus 2.18 ± 0.74 mg/liter for CFX alone, p < .01).

The involvement of adrenoceptors was tested with drugs reputed to block or stimulate α2, α1 and β adrenoceptors. Idazoxan (0.3 mg/kg, i.v.), an α2 adrenoceptor antagonist, did not significantly change basal CFX absorption when it was injected before CFX in the absence of NFP (Table 1). However, idazoxan injected before NFP + CFX tended to reduce by more than 50% (Fig. 6) the mean increase in plasma CFX produced by NFP, although this was not significant. Clonidine, an α2 adrenoceptor agonist, did not change plasma CFX at the dose of 100 mg/kg but approximately doubled plasma CFX at the dose of 300 μg/kg (p < .05, Fig 6). We tested that the clonidine effect was correlated to its α2 adrenoceptor agonist properties by using the α2 adrenoceptor antagonist yohimbine. As shown on Fig. 7, yohimbine (1 mg/kg) suppressed the clonidine-induced increase of CFX absorption. Thus, although an α2 adrenoceptor antagonist tended to reduce the effect of NFP, an α2 adrenoceptor agonist produced an increase of CFX absorption like NFP.

To determine whether clonidine stimulates the active portion of CFX absorption, we again checked the clonidine effect while competitively antagonizing the di- and tripeptide transporter by a large concentration of dipeptides (85 mM). In presence of dipeptides, the effect of clonidine on CFX absorption was abolished (Fig. 7). However, a fraction of
basal and clonidine-stimulated CFX absorption remained unchanged (Fig. 7), which was likely to correspond to passive absorption.

Prazosin (0.5 mg/kg, i.v.), an alpha-1 adrenoceptor antagonist, did not change the effect of NFP on CFX absorption (Fig. 8). The alpha-1 adrenoceptor agonist phenylephrine (100 μg/kg/h, i.v.) also had no effect on the absorption of CFX perfused alone. However, an unexpected finding was that the plasma CFX level was enhanced by 49% \( (p < .05) \) in the group receiving prazosin + CFX, with respect to the group CFX alone (Table 1).

Propranolol (1 mg/kg, i.v.), a beta adrenoceptor antagonist, had no effect on the absorption of CFX perfused alone (Table 1). However, propranolol tended to reduce by approximately 60% the mean increase of plasma CFX produced by NFP (Fig. 9), although this was not significant. Isoproterenol, a beta adrenoceptor agonist, approximately doubled plasma CFX at 60 min when administered at the CFX perfusion in doses equal to or larger than 0.1 μmol/kg/h.

Because the alpha-2 and beta antagonists idazoxan and propranolol reduced but did not suppress the increase of CFX absorption produced by NFP, we tested their combination to search for an additive effect. Indeed, when propranolol and idazoxan were combined, the effect of NFP was totally abolished (4.30 ± 0.60 mg/liter versus 1.90 ± 0.12 mg/liter, \( p < .05 \)).

**Discussion**

In the present study, CFX plasma concentration was approximately doubled when NFP was administered before CFX either as an intrajejunal perfusion or an i.v. injection. The effect was observed both in portal and systemic plasma, confirming the pharmacokinetic data (Welles et al., 1968), indicating that the liver had no first-pass effect on the circulating levels of the drug and that CFX undergoes no metabolic transformation in vivo. In addition, NFP also increased CFX urinary elimination, demonstrating that the plasma concentration of CFX can be taken in the conditions of our study as an index of CFX absorption in the perfused jejunal loop. Entero-hepatic recycling could not participate in the results of this study, because the bile duct was ligated. The present data obtained with CFX confirm the general effect of NFP on peptidomimetic drugs transported via the proton-dependent di- and tripeptide transporter, which has been previously demonstrated in humans on the bioavailability of amoxicillin (Westphal et al., 1990) and of cefixime (Duverne 1999).
et al., 1992), and in the rat on the plasma concentration of cefixime (Harcouët et al., 1997).

This effect was not specific to NFP, but was reproduced by diltiazem, a drug with different molecular structure, but reputed also to block L-type calcium channels. This finding strongly suggests that calcium channel blocking activity is involved in the NFP effect.

In addition, NFP modulation of CFX transport suggests that the control mechanisms demonstrated in the present studies affect the function of the proton-stimulated di- and tripeptide intestinal transporter. It has indeed been clearly shown with in vitro models that CFX is actively transported through this system (Okano et al., 1986; Dantzig and Bergin, 1990; Dantzig et al., 1992; Sugawara et al., 1992; Hidalgo et al., 1993; Gochoco et al., 1994; Leibach and Ganapathy, 1996), and that the passive membrane permeability of CFX, which is independent of this transporter, is 0.0, compared with 0.18 for cefixime and 0.76 for amoxicillin (Bai et al., 1992), suggesting that 100% of the transported drug crosses the mucosa via the di- and tripeptide transporter. In the present experiments, however, CFX transport was inhibited maximally by only 60% in the presence of 170 mM of dipeptides, suggesting that the competition demonstrated in vivo in the rat is less complete than in vitro on cultured cell models. However, the NFP-stimulated fraction of CFX transport was totally inhibited in the presence of 85 mM of dipeptides, suggesting that the transport increase produced by NFP treatment was totally dependent of the di- and tripeptide transporter activity. This control by NFP is very likely indirect, as discussed below.

In a previous study, Harcouët et al. (1997) showed that the effect of NFP on the absorption of cefixime could be demonstrated only in vivo, and was not found on any of the in vitro models they used (Ussing chambers, cell cultures, and brush border membrane vesicles). Furthermore, their data indicated that neither a hemodynamic effect of NFP nor its effect on intestinal motility is responsible for its proabsorptive effect. NFP has been shown to decrease intestinal motility by inhibiting migrating motor complexes in the rat single-pass small intestinal perfusion model (Thallander et al., 1993). We thus thought that intestinal sensory neurons are likely to be influenced by calcium channel blocking agents such as NFP (Perney et al., 1986; Fox et al., 1987). We thus thought that intestinal sensory neurons might be a target of NFP in our model, and might be involved in the control of CFX transport. The acute perfusion of capsaicin in the jejunal lumen did indeed stimulate CFX transport, suggesting that activation of extrinsic sensory terminals in the gut can control the transporter function. Because capsaicin was locally administered on the mucosa, it is likely that mucosal endings were involved. It is not yet known whether activation of these endings affects directly the transporter via the local effenter function of a single sensory neuron, or if the effect is indirect through a complex neuronal network. Lidocaine, which blocks sensory fibers, but also diverse small unmyelinated fibers of the gut, totally suppressed the effect of NFP, confirming its dependence upon local neuronal fibers.

Several results indicate that adrenoceptors are important to control CFX transport. The alpha-2 antagonist idazoxan tended to decrease the effect of NFP, whereas the alpha-2 agonist clonidine reproduced a stimulation of CFX transport analogous to the effect of NFP. One may thus suspect a proabsorptive effect of alpha-2 adrenoceptors on CFX trans-
port. Previous studies clearly demonstrated that alpha-2 adrenoceptor agonists potently stimulate the net absorption of electrolytes and water (Chang et al., 1982; Nakaki et al., 1982; Cox and Cuthbert, 1989), but no study has to date suggested that they might also affect peptide transport. Because dipeptides abolished the effect of clonidine, it seems that alpha-2 adrenoceptor stimulation can enhance the di- and tripeptide transporter activity. Apart from alpha-2 adrenoceptors, clonidine has also been reported to bind to non-adrenergic imidazoline receptors (Guyenet, 1997). Yohimbine, however, is reputed to have poor affinity for imidazoline binding sites. Because the effect of clonidine was suppressed by yohimbine, it seems indeed related to alpha-2 adrenoceptors.

**Alpha-1 adrenoceptors** are usually thought to little affect electrolyte transport, although an opposite effect of alpha-1 and alpha-2 mechanisms has been claimed (Cotterel et al., 1984). In the present study, the alpha-1 antagonist prazosin did not change the effect of NFP, and the alpha-1 agonist phenylephrine did not increase CFX transport. However, prazosin increased CFX transport, in a fashion similar to clonidine. The mechanism of this effect is not clear, but we may hypothesize that prazosin might reveal the alpha-2 tone of endogenous norepinephrine by removing the balancing effect of an opposite alpha-1 mechanism. Because intestinal epithelial cells have been reported to express alpha-1- and -2 adrenoceptors (Nakaki et al., 1983; Cotterel et al., 1984), clonidine and prazosin might act directly on enterocytes. In this cell type, the stimulation of alpha-2 adrenoceptors reduces the vasointestinal peptide- or prostaglandin E2-induced cAMP production that induces intestinal secretion. This effect on cAMP could indirectly regulate the di- and tripeptide transporter, because increased cAMP levels in the cell have been shown to inhibit the activity of the H+/peptide cotransporter through an indirect activation of protein kinase C (Brandsch et al., 1994).

Apart from enterocytes, alpha-2 adrenoceptors are also present on enteric neurons (Cooke and Reddix, 1994) and might also be involved. Norepinephrine is normally released from extrinsic sympathetic postganglionic axons (Cooke and Reddix, 1994). NFP might increase norepinephrine release from these fibers and thus increase CFX absorption. Ida-zoxan would oppose this effect by blocking the alpha-2 tone and idazoxan suggested that NFP used in part alpha-2 and in part beta endogenous pathways, because it was necessary to combine both antagonists to totally suppress the NFP effect. We thus propose that intestinal sympathetic neurons represent a target for NFP in our model, and can be involved in the control of CFX transport.

In summary, CFX absorption depends on the activity of intramural neurons. Noradrenergic mechanisms and sensory neurons appear important, whereas other neurons might also modulate the function of the di- and tripeptide transport vector via a complex neural circuitry.

**References**


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